(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 April 2001 (05.04.2001)

PCT

(10) International Publication Number
WO 01/23608 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/EP00/09459

(22) International Filing Date:
25 September 2000 (25.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/156,171 27 September 1999 (27.09.1999) US

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(81) Designated States *(national)*: CA, JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, NL, PT, SE).

Published:

— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/23608 A2

(54) Title: HYBRIDIZATION PROBES WHICH SPECIFICALLY DETECT STRAINS OF THE GENERA MICROBISPORA, MICROTETRASPORA, NONOMURIA AND PLANOBISPORA

(57) Abstract: A method is provided herein which allows for the specific detection of bateria from the genera *Microbispora*, *Microtetraspora*, *Nonomuria* and *Planobispora*. This method makes use of four specific nucleic acid probes also provided herein that are complementary to a conserved region of the 16S rRNA genes present within said strains. The probes permit rapid detection of the strains in an accurate and reproducible fashion.

HYBRIDIZATION PROBES WHICH SPECIFICALLY DETECT STRAINS OF THE GENERA MICROBISPORA, MICROTETRASPORA, NONOMURIA AND PLANOBISPORA

FIELD OF THE INVENTION

The present invention relates to nucleic acid probes which specifically detect strains of the genera *Microbispora, Microtetraspora, Nonomuria* and *Planobispora*. Said probes are derived from the region coding for the mature 16S ribosomal RNA (rRNA) of the respective strains. The invention also relates to assays using these probes.

BACKGROUND OF THE INVENTION

Although much progress has been made in the last decade with regards to the identification of microorganisms, the procedures in use are often still laborious, non-sensitive and not specific. Traditional methods only permit the identification of those microorganisms that can be cultivated in the laboratory. This makes an accurate description of the composition of natural microbial communities very difficult. It is generally accepted that less than 20% of the microorganisms in nature have been discovered and, further, that the development of new culture-independent methods for studying the composition of microbial communities is well needed (Ward, D.M. *et al.*, 1990 *Nature*, 345:63-64).

Many of the pitfalls intrinsic to traditional methods have been overcome through the use of nucleic acid probes. These nucleic acid probes, made of genomic DNA, plasmids, riboprobes or synthetic oligonucleotides, are designed to target genomic DNA or certain RNA species present in biological samples. The use

of synthetic oligonucleotides as probes is largely preferred because oligonucleotides can be rapidly synthesized in large amounts using chemical methods, have a long shelf-life, and are easy to purify and label.

The sequence of 16S rRNA, a common but distinctive cellular element, has been largely useful for this purpose, and has revealed the presence of numerous uncultured microorganisms in natural communities (Ward, D.M., *et al.*, *supra*; Giovanni, S.J. *et al.*, 1990 *Nature* 345:60-63; Barry, T. *et al.*, 1991 In PCT *Methods and Applications*, Cold Spring Harbour, pp 51-56; Mehling, A *et al.*, 1995 *Microbiology* 141:2139-2147; and Rheims, H. *et al.*, 1996 *Microbiology* 142:2863-2870).

16S and 23S rRNA genes are very often used for probe development given that sequences can readily be obtained using described methods and it is known that variable regions exist within these highly conserved genes which can be used for species-specific detection. Species-specific probes have been described for a large number of microorganisms. Universal probes for the detection of bacteria are also known (Giovannoni, S.J. *et al.*, 1988 *J. Bacteriol.* 170:720-726, and Barry, T *et al.*, 1991 *supra*).

Modern methods, based on the 16S rRNA sequence have permitted the establishment of new evolutionary trees. From these analyses, the class *Actinobacteria* (Stackebrandt *et al.*, 1997 *Int. J. Syst. Bacteriol.* 47:479-491) (comprising aerobic, gram-positive bacteria which form branching, usually non-fragmenting hyphae, and asexual spores borne on aerial mycelia) is shown to exhibit extraordinary complexity, encompassing many different genera clustered in at least 35 families.

Currently the art is silent as to a simple yet robust method to discriminate between close groups of Actinomycetes that occur in natural isolates, particularly for those bacteria that develop aerial mycelia prior to release of their spores. It would, thus, be desirable to have nucleic acid probes that could be used in fast, accurate assays to detect specific strains of Actinomycetes. Of particular interest in this respect are the *Microbispora, Microtetraspora, Nonomuria* (a new genus recently proposed: Zhensui, Z *et al.*, 1998 *Int. J. Syst. Bacteriol.* 48:411-422) and *Planobispora* strains that belong to these genera.

SUMMARY OF THE INVENTION

This invention relates to nucleic acid probes which hybridize under hybridization conditions to nucleic acids encoding a portion of the 16S rRNA of bacteria belonging to genera selected from the group consisting of *Microbispora*, *Microtetraspora*, *Nonomuria* and *Planobispora*.

Another aspect of this invention is a method for detecting the presence of bacteria belonging to genera selected from the group consisting of *Microbispora*, *Microtetraspora*, *Nonomuria* and *Planobispora*, comprising:

(a) contacting the sample with a nucleic acid probe specific for the genus of interest; wherein said probe hybridizes to nucleic acid encoding 16S rRNA in said genus;

(b) imposing hybridization conditions; and

(c) determining if hybridization has occurred.

Yet another aspect of this invention is a kit for the detection of bacteria from the genera *Microbispora*, *Microtetraspora*, *Nonomuria* and *Planobispora*, comprising:

(a) a specific probe for bacteria of the genus *Microbispora* from 10 to 285 nucleotides in length which is complementary to or homologous with at least 90% of a nucleic acid sequence comprising *Microbispora* nucleic acids corresponding to base pairs 528 to 547 of *Microbispora aerata* DNA encoding the mature 16S rRNA molecule (Wang, Y *et al.*, 1996 *Int. J. Syst. Bacteriol.* 46:658-663);

(b) a specific probe for bacteria of the genus *Microtetraspora* (including the strain *Microtetraspora pusilla*, despite of being classified within the genus *Nonomuria*) from 10 to 285 nucleotides in length which is complementary to or homologous with at least 90% of a nucleic acid sequence comprising *Microtetraspora* nucleic acids corresponding to base pairs 523 to 543 of *Microtetraspora glauca* DNA encoding the mature 16S rRNA molecule (Wang, Y *et al.*, *supra.*);

(c) a specific probe for bacteria of the genus *Nonomuria* (excluding the strain *Microtetraspora pusilla*) from 10 to 285 nucleotides in length which is complementary to or homologous with at least 90% of a nucleic acid sequence comprising *Nonomuria* nucleic acids corresponding to base pairs 527 to 555 of *Nonomuria ferruginea* (formerly *Microtetraspora ferruginea*) DNA encoding the mature 16S rRNA molecule (Wang, Y. *et al.*, *supra*); and

(d) a specific probe for bacteria of the genus *Planobispora* from 10 to 285 nucleotides in length which is complementary to or homologous with at least

90% of a nucleic acid sequence comprising *Planobispora* nucleic acids corresponding to base pairs 537 to 558 of *Planobispora longispora* DNA encoding the mature 16S rRNA molecule (Wang, Y. *et al., supra*).

The kit may additionally comprise reagents, compositions, instructions, disposable hardware and suitable packaging.

BRIEF DESCRIPTION OF THE DRAWINGS

Not Applicable

DETAILED DESCRIPTION OF THE INVENTION

A method is provided herein which allows for the specific detection of bacteria from the genera *Microbispora, Microtetraspora, Nonomuria* and *Planobispora*. This method makes use of four specific nucleic acid probes also provided that are complementary to a conserved region of the 16S rRNA genes present within said strains. The probes permit rapid detection of the strains in an accurate and reproducible fashion.

As used throughout this application and claims, the term "probe" will refer to synthetic or biologically produced nucleic acids, between 10 and 285 base pairs in length which contain specific nucleotide sequences that allow specific and preferential hybridization under predetermined conditions to target nucleic acid sequences, and optionally contain a moiety for detection or for enhancing assay performance. A minimum of ten nucleotides is generally necessary in order to statistically obtain specificity and to form stable hybridization products, and a maximum of 285 nucleotides generally represents an upper limit for length in which reaction parameters can be easily adjusted to determine mismatched sequences and preferential hybridization. Probes may optionally contain certain constituents that contribute to their proper or optimal functioning under certain assay conditions. For example, probes may be modified to improve their resistance to nuclease degradation (e.g., by end capping), to carry detection ligands (e.g., fluorescein, ^{32}P, biotin, etc.) or to facilitate their capture onto a solid support (e.g., poly-deoxyadenosine "tails").

"Preferential hybridization" or "hybridizing preferentially" means that hybridization with the intended target nucleic acid results in a hybridization reaction product which is more stable than any hybridization reaction product resulting from hybridization with a non-target nucleic acid under identical conditions. It is well within the skill of the ordinary artisan to compare stability of hybridization reaction

products and evaluate which one is more stable, i.e., determine which one has bound "preferentially."

The terms "homology" and "homologous to" are meant to refer to the degree of similarity between two or more nucleic acid sequences and is not meant to imply any taxonomic relatedness between organisms. The degree of similarity is expressed as a percentage, i.e., 90% homology between two sequences will mean that 90% of the bases of the first sequence are identically matched to the bases of the second sequence.

"Specific" means that a nucleotide sequence will hybridize to a predetermined target sequence and will not substantially hybridize to a non-target sequence.

"Specifically discriminate" means that a probe will substantially hybridize to a predetermined target sequence and will not substantially hybridize to a non-target sequence.

"Hybridization" is a process by which, under predetermined reaction conditions, two partially or completely complementary strands of nucleic acid are allowed to come together in an antiparallel fashion to form a double-stranded nucleic acid with specific and stable hydrogen bonds, following explicit rules pertaining to which nucleic acid bases may pair with one another.

"Substantial hybridization" means that the amount of hybridization observed will be such that one observing the results would consider the result positive in a clinical setting. Data which is considered "background noise" is not substantial hybridization.

"Stringent hybridization conditions" means approximately 35°C to 65°C in a salt solution of approximately 0.9 molar NaCl. Stringency may also be governed by such reaction parameters as the concentration and type of ionic species present in the hybridization solution, the types and concentrations of denaturing agents present, and the temperature of hybridization. Generally as hybridization conditions become more stringent, longer probes are preferred if stable hybrids are to be formed. As a rule, the stringency of the conditions under which hybridization is to take place will dictate certain characteristics of the preferred probes to be employed. Such relationships are well understood and can be readily manipulated by those skilled in the art.

In designing a probe for identification purposes, it is preferred that the probe be as specific as necessary (i.e., it should not cross-react with undesired nucleic

acids) and highly sensitive (i.e., most, if not all, strains of the organism to be detected should react with the probe). Hence, the preferred target sequences should have the following characteristics:

(a) the sequence should be present in the genome of each strain of the microorganism concerned; and

(b) the evolutionary diversity of the sequence should be such that, on the one hand, there is sufficient sequence-diversity to allow differentiation of the species concerned from other closely related species and, on the other hand, sufficient sequence-conservation to allow detection of the strain of concern with the probe used.

Comparison and alignment of 16S rDNA sequences present in databases for the many actinomycetes and bacteria of the genera *Microbispora, Microtetraspora, Nonomuria* and *Planobispora* confirmed the existence of the so-called variable regions that appear in all 16S rDNA of actinomycetes, and allowed for the identification of a particular region within the strains above which seemed suitable to derive probes that would be individually specific for bacteria of each of the four genera. Sequence comparison and analysis were carried out using programs from the UWGCG package (Version 9.0, December 1996) and the Edit-View 1.0 DNA sequencer viewer (Applied Biosystems). 16S rDNA sequences were obtained from the NCBI Genbank database. Probes were checked against all 16S rRNA available via Ribosomal Database Project World Wide Web server (Bonnie, L. *et al.*, 1999 A new version of the RDP (Ribosomal Database Project), *Nucleic Acids Res.* 27:171-173).

As a result of the sequence comparison, two primers were designed. The first is designated GNR-N: 5' GCCAGCAGCCGCGGTAATACGT 3' (SEQ ID NO:1) corresponding to base pairs 489-510 from *Streptomycetes ambofaciens* DNA encoding mature 16S rRNA (Pernodet *et al.*, 1989 *Gene* 79:33-46). The second is designated GNR-R: 5' CTACCAGGGTATCTAATCCTGTT 3' (SEQ ID NO: 2) corresponding to base pairs 752-774 from *Streptomycetes ambofaciens* DNA encoding mature 16S rRNA (Pernodet *et al.*, *supra.*). Both of these can be used as primers in polymerase chain reaction technology to amplify the DNA region in order to obtain a preferred probe of this invention. Designated large quantities of the probe can be generated using known PCR techniques such as those in U.S. patents 4,683,202 and 4,683,195.

A preferred probe CNB-MIB.1 was derived from a variable region of the 16S rRNA molecule. It comprises the sequence 5'

AGCCCGTGGCTTAACTACGG 3' (SEQ ID NO:3), which is DNA corresponding to base pairs 528-547 from *Microbispora aerata* DNA encoding mature 16S rRNA. In accordance with this invention, probes similar to CNB-MIB.1 may be made by increasing or decreasing the length of CNB-MIB.1. For a longer probe, it is preferred that additional nucleotides (either 3' or 5') be those of the corresponding DNA of *Microbispora* strains encoding mature 16S rRNA.

A preferred probe CNB.MIC.2 was derived from a variable region of the 16S rRNA molecule. It comprises the sequence 5'AGCTTAGGGCTTAACCCTAGG 3' (SEQ ID NO:4), which is maduromycetes DNA corresponding to base pairs 523-543 from *Microtetraspora glauca* DNA encoding mature 16S rRNA. In accordance with this invention, probes similar to CNB-MIC.2 may be made by increasing or decreasing the length of CNB-MIC.2. For a longer probe, it is preferred that additional nucleotides (either 3' or 5') be those of the corresponding DNA of *Microtetraspora* strains encoding mature 16S rRNA.

A preferred probe CNB-NOM.1 was derived from a variable region of the 16S rRNA molecule. It comprises the sequence 5' GCAGCTTAACTGCGGGTCTGCGGTGGATA 3' (SEQ ID NO:5), which is *Nonomuria ferruginea* DNA corresponding to base pairs 527-555 from the DNA encoding *Nonomuria ferruginea* mature 16S rRNA. In accordance with this invention, probes similar to CNB-NOM.1 may be made by increasing or decreasing the length of CNB-NOM.1. For a longer probe, it is preferred that additional nucleotides (either 3' or 5') be those of the corresponding DNA of *Nonomuria* strains encoding mature 16S rRNA.

A preferred probe CNB-PLAB.1 was derived from a variable region of the 16S rRNA molecule. It comprises the sequence 5' TCGGATGTGAAAGCCTGCAGCT 3' (SEQ ID NO:6), which is *Planobispora longispora* DNA corresponding to base pairs 537-558 from the DNA encoding *Planobispora longispora* mature 16S rRNA. In accordance with this invention, probes similar to CNB-PLAB.1 may be made by increasing or decreasing the length of CNB-PLAB.1. For a longer probe, it is preferred that additional nucleotides (either 3' or 5') be those of the corresponding DNA of *Planobispora* strains encoding mature 16S rRNA.

One embodiment of this invention is a nucleic acid, designated CNB-MIB.1, which provides specific binding to chromosomal DNA encoding the mature part of the 16S rRNA molecule of bacteria from the genus *Microbispora* and does not

substantially hybridize to the equivalent chromosomal region from bacteria belonging either to closely related actinomycetes taxa or to other bacteria.

One embodiment of this invention is a nucleic acid, designated CNB-MIC.2, which provides specific binding to chromosomal DNA encoding the mature part of the 16S rRNA molecule of bacteria from the genus *Microtetraspora* and does not substantially hybridize to the equivalent chromosomal region from bacteria belonging to closely related actinomycetes or to other bacteria.

One embodiment of this invention is a nucleic acid, designated CNB-NOM.1, which provides specific binding to chromosomal DNA encoding the mature part of the 16S rRNA molecule of bacteria from the genus *Nonomuria* and does not substantially hybridize to the equivalent chromosomal region from bacteria belonging to closely related actinomycetes or to other bacteria.

One embodiment of this invention is a nucleic acid, designated CNB-PLAB.1, which provides specific binding to chromosomal DNA encoding the mature part of the 16S rRNA molecule of bacteria from the genus *Planobispora* and does not substantially hybridize to the equivalent chromosomal region from bacteria belonging to closely related actinomycetes or to other bacteria.

The preferred probes of this invention generally contain from at least about 10 nucleotides to about 285 nucleotides (the maximum number of nucleotides of the mature region of the genes coding for the 16S rRNA). More preferably, the probe will contain from about 16 nucleotides to about 285 nucleotides resulting from the PCR amplification of a DNA fragment comprising a DNA sequence hybridizing with the probes CNB-MIB.1, CNB-MIC.2, CNB-NOM.1 and CNB-PLAB.1.

The invention also relates to probes for use in hybridization assays, which use an oligonucleotide sufficiently complementary to hybridize to a sequence of chromosomal DNA region encoding the mature 16S rRNA from bacteria of the genera *Microbispora, Microtetraspora, Nonomuria* and *Planobispora*, yet not complementary enough to hybridize to the equivalent region from far-related Gram positive bacteria (e.g., *Bacillus subtilis*).

A particularly preferred assay in accordance with this invention is a Southern blot. One probe which can be used for a Southern blot assay is about 285 bp long, obtained by PCR amplification of the DNA fragment obtained by use of the two primers GNR-N and GNR-R. The Southern blot, or dot blot assay can be conducted using well known procedures. Generally, it involves the steps of immobilizing a target nucleic acid or population of nucleic acids on a filter such as nitrocellulose,

nylon or other derivatized membranes which are readily commercially available. The immobilized nucleic acids are then tested for hybridization under predetermined stringency conditions with the probe of interest. Under stringent conditions probes with nucleotide sequences with greater complementary to the target will exhibit a higher level of hybridization than probes whose sequences have less homology. Hybridization can be detected in a number of ways. For example, the probe can be isotopically labeled with the addition of a ^{32}P-Phosphorous moiety to the 5'-end of the oligonucleotide by the conventional polynucleotide kinase reaction. After hybridization has occurred, non-hybridized probe is removed by washing. The filters are exposed to x-ray film and the intensity of the hybridization signals is evaluated.

Another preferred assay in accordance with this invention is PCR. The probes CNB-MIB.1, CNB-MIC.2 and CNB-PLAB.1 may be used as primers in a Polymerase Chain Reaction in combination with the primer RVR.3 (SEQ ID NO:7, 5' AAGCTTTTAAGCAACATGCTCCGCCG 3') so as to specifically amplify a DNA fragment of about 370 bp, only when genomic DNA from the strains of the genus *Microbispora, Microtetraspora* and *Planobispora* respectively are used. The temperatures of annealing for specific detection are 50°C for CNB-MIB.1 and 60°C for CNB-MIC.2 and CNB-PLAB.1.

The probes of this invention may be chemically synthesized using commercially available methods and equipment. For example, the solid phase phosphoramidite methods can be used to produce short oligonucleotides between 15 and 30 nucleotides long. Preferably, short DNA oligonucleotides are synthesized chemically using any of the Applied Biosystems DNA Synthesizers with reagents supplied by the same company. Chemically synthesized oligonucleotides can be obtained from Boehringer Mannheim.

Actinomycetes strains used in this invention may be obtained from the ATCC collection and are included in the following table:

Strain Name	ATCC Number	Strain Name	ATCC Number
Actinomadura citrea	ATCC 27887	*Actinomadura viridis*	ATCC 27103
Actinomadura coerulea	ATCC 33576	*Microbispora aerata*	ATCC 15448
Actinomadura cremea	ATCC 33577	*Microbispora amethystogenes*	ATCC 15740
Actinomadura spadix	ATCC 27298	*Microbispora diastatica*	ATCC 33325
Nonomuria spiralis	ATCC 35114	*Microbispora echinospora*	ATCC 27300
Nonomuria fastidiosa	ATCC 33516	*Microbispora parva*	ATCC 33326
Nonomuria ferruginea	ATCC 35575	*Microbispora rosea*	ATCC 12950
Nonomuria helvata	ATCC 27295	*Microtetraspora flexuosa*	ATCC 35864
Actiniomadura livida	ATCC 33578	*Microtetraspora fusca*	ATCC 23058
Actinomadura madurae	ATCC 19425	*Microtetraspora glauca*	ATCC 23057
Actinomadura malachitica	ATCC 27888	*Microtetraspora niveoalba*	ATCC 27301
Nonomuria pusilla	ATCC 27296	*Planobispora rosea*	ATCC 23866
Nonomuria roseola	ATCC 33579	*Planomonospora parontospora*	ATCC 23863
Nonomuria rubra	ATCC 27031	*Streptosporangium roseum*	ATCC 12428
Nonomuria salmonea	ATCC 33580	*Streptosporangium vulgare*	ATCC 33329

The following stremptomycetes strains may be used in this invention: *Streptomyces ambofaciens, S. antibioticus, S. cinnamonensis, S. coelicolor* A3 (2), *S. fradiae, S. lividans* TK21, *S. nataliensis, S. peucetius, S. violascens* and *Streptomyces sp.*

Procedures used for the growth and manipulation of the bacteria related to this invention and general DNA manipulation are as described (Hopwood *et al.*, 1985 *Genetic Manipulation of Streptomyces. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). For DNA extraction, bacteria are grown in LB or YEME media at the ATCC recommended temperatures. Chromosomal DNA is purified from cultures growing in late exponential phase as described for maize (Dellaporta *et al.*, 1985 "Maize DNA Miniprep," *Molecular Biology of Plants – a Laboratory Course Manual.* Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory, N.Y. USA. Pp. 36-37), as adapted for *Streptomyces* (Mehling *et al.*, 1995 *Microbiology* 141:2139-2147).

The following non-limiting examples are presented to better illustrate the invention.

EXAMPLE 1

Identification of *Microbispora, Microtetraspora, Nonomuria* and *Planobispora* Strains by Southern Analysis of PCR Amplified DNA

Strains of bacteria were obtained and grown until mid-logarithmic phase to validate the usefulness of the CNB-MIB.1, CNB-MIC.2, CNB-NOM.1 and CNB-PLAB.1 probes to identify bacteria of the genera *Microbispora, Microtetraspora, Nonomuria* and *Planobispora*, respectively. Genomic DNA was prepared as follows: Approximately 0.5-1.0 g mycelia were resuspended in 2 ml lysis buffer (NaCl 0.1M, EDTA 50 mM, pH 8.0) containing glass beads (3 mm diameter) and the suspension was vortexed for 2 minutes before adding 2 ml of lysis buffer plus 10-15 mg lysozyme and 50 $\mu g\ ml^{-1}$ Rnase Dnase-free. The suspension was incubated for 30-80 minutes at 37°C. After the addition of 500 µl 10% SDS (w/v), the solution was incubated at 37°C for 15 min. The glass beads were removed and the DNA extracted four times with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once more with 1 volume of chloroform. The extracted DNA was ethanol precipitated, dried and resuspended in 500 µl distilled water.

Chromosomal DNA from all the strains listed above was used for PCR amplification using primers GNR.N: 5' GCCAGCAGCCGCGGTAATACGT 3' (SEQ ID NO:1) and GNR.R: 5' CTACCAGGGTATCTAATCCTGTT 3' (SEQ ID NO:2). Approximately 0.5-1.0 µg genomic DNA template was used with 280 ng of each primer in a final reaction volume of 100 µl of an incubation buffer containing

16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCL (pH8.8), 0.1% Tween-20 and 1mM $MgCl_2$. Amplifications were performed in automated thermocyclers by incubation at 95°C (5 min) followed by 30 cycles of incubation at 95°C (1 min), 55°C (1 min) and 72°C (1 min) in the presence of one unit of Eco Taq polymerase (Ecogen).

The resulting about 285 bp long amplified DNA fragments were fractionated by electrophoresis in 1.5% agarose gels. The fractionated DNA fragments were transferred to Hybond N+ membranes (Amersham, plc.) by capillary transfer for 16 h. The DNA immobilized in the solid support was then washed with a hybridization buffer containing 5 x SSC, 5 x Denhardt's solution and 0.5% SDS and set to hybridize with 10 pmol of the radioactively labeled probes CNB-MIB.1, CNB-MIC.2, CNB-NOM.1 and CNB-PLAB.1 in the same buffer for 16 h at 50°C, 50°C, 65°C, and 60°C, respectively. The solid supports were then washed three times with 1 x SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2) and 0.5% SDS at the hybridization temperature. The solid supports were then set to exposure in X-ray films at -80°C prior to being developed.

EXAMPLE 2

Detection of Bacteria from the Genera *Microbispora, Microtetraspora, Nonomuria* and *Planobispora* by PCR

The probes CNB-MIB.1, CNB-MIC.2 and CNB-PLAB.1 can be used as forward primers for PCR in order to specifically amplify fragments from the target strains only. As reverse primer the conserved oligonucleotide RVR.3 (SEQ ID NO:7) can be used in all cases.

Approximately 0.5-1.0 µg genomic DNA template of representative strains of each genera of bacteria listed above was used with 280 ng of each primer in a final reaction volume of 100 µl of an incubation buffer containing 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH8.8), 0.1% Tween-20 and 1 mM $MgCl_2$. Amplifications were performed in automated thermocyclers by incubation at 95°C (5 min) followed by 30 cycles of incubation at 95°C (1 min), temperature of annealing for specific detection are 50°C for CNB-MIB.1 and 60°C for CNB-MIC.2 and CNB-PLAB.1 (1 min) and 72°C (1 min) in the presence of one unit of Eco Taq polymerase (Ecogen).

DNA fragments of the expected size were obtained only when the target organisms DNA were present in the reaction.

SEQUENCE LISTING

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<120> HYBRIDIZATION PROBES WHICH SPECIFICALLY DETECT STRAINS OF THE GENERA MICROBISPORA, MICROTETRASPORA, NONOMURIA AND PLANOBISPORA

<130> PCT 20516

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<151> 1999-09-27

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WHAT IS CLAIMED:

1. A nucleic acid probe which hybridizes under hybridization conditions to nucleic acid encoding a portion of 16S rRNA of bacteria belonging to genera selected from the group consisting of *Microbispora*, *Microtetraspora*, *Nonomuria* and *Planobispora*.

2. A probe according to claim 1 which hybridizes to nucleic acid encoding a portion of 16S rRNA of bacteria belonging to the genus *Microbispora*.

3. A probe according to claim 2 which is DNA.

4. A probe according to claim 3 which is between 10 and 285 base pairs.

5. A probe according to claim 4 which is complementary to or homologous with at least 90% of a nucleic acid sequence comprising a *Microbispora* nucleic acid corresponding to base pairs 528-547 of *Microbispora aerata* DNA encoding mature 16S rRNA.

6. A probe according to claim 4 which comprises 5' AGCCCGTGGCTTAACTACGG 3' (SEQ ID NO:3).

7. A probe according to claim 1 which hybridizes to nucleic acid encoding a portion of 16S rRNA of bacteria belonging to the genus *Microtetraspora*.

8. A probe according to claim 7 which is DNA.

9. A probe according to claim 8 which is between 10 and 285 base pairs.

10. A probe according to claim 9 which is complementary to or homologous with at least 90% of a nucleic acid sequence comprising a *Microtetraspora* nucleic acid corresponding to base pairs 523-543 of *Microtetraspora glauca* DNA encoding mature 16S rRNA.

11. A probe according to claim 9 which comprises 5' AGCTTAGGGCTTAACCCTAGG 3' (SEQ ID NO:4).

12. A probe according to claim 1 which hybridizes to nucleic acid encoding a portion of 16S rRNA of bacteria belonging to the genus *Nonomuria*.

13. A probe according to claim 12 which is DNA.

14. A probe according to claim 13 which is between 10 and 285 base pairs.

15. A probe according to claim 14 which is complementary to or homologous with at least 90% of a nucleic acid sequence comprising a *Nonomuria* nucleic acid corresponding to base pairs 527-555 of *Nonomuria ferruginea* DNA encoding mature 16S rRNA.

16. A probe according to claim 14 which comprises 5' GCAGCTTAACTGCGGGTCTGCGGTGGATA 3' (SEQ ID NO:5).

17. A probe according to claim 1 which hybridizes to nucleic acid encoding a portion of 16S rRNA of bacteria belonging to the genus *Planobispora*.

18. A probe according to claim 17 which is DNA.

19. A probe according to claim 18 which is between 10 and 285 base pairs.

20. A probe according to claim 19 which is complementary to or homologous with at least 90% of a nucleic acid sequence comprising a *Planobispora* nucleic acid corresponding to base pairs 537-558 of *Planobispora longispora* DNA encoding mature 16S rRNA.

21. A probe according to claim 19 which comprises 5' TCGGATGTGAAAGCCTGCAGCT 3' (SEQ ID NO:6).

22. A method for detecting the presence of bacteria belonging to genera selected from the group consisting of *Microbispora*, *Microtetraspora*, *Nonomuria* and *Planobispora*, in a sample comprising:

(a) contacting the sample with a nucleic acid probe specific for the genus of interest; wherein said probe hybridizes to nucleic acid encoding 16S rRNA in said genus;

(b) imposing hybridization conditions; and

(c) determining if hybridization has occurred.

23. A method according to claim 22 wherein the genus is *Microbispora*.

24. A method according to claim 23 further comprising the step of lysing bacteria in the sample prior to step (a).

25. A method according to claim 24 wherein the probe is a radioactively labeled probe.

26. A method according to claim 25 wherein hybridization conditions are stringent hybridization conditions.

27. A method according to claim 26 wherein said probe comprises nucleic acids selected from the group consisting of:

(a) nucleic acids comprising SEQ ID NO:3; and

(b) nucleic acid comprising a 285 bp amplification product made by PCR amplification using primers SEQ ID NO:1 and SEQ ID NO:2.

28. A method according to claim 22 wherein the genus is *Microtetraspora*.

29. A method according to claim 28 further comprising the step of lysing bacteria in the sample prior to step (a).

30. A method according to claim 29 wherein the probe is a radioactively labeled probe.

31. A method according to claim 30 wherein hybridization conditions are stringent hybridization conditions.

32. A method according to claim 31 wherein said probe comprises nucleic acids selected from the group consisting of:

(a) nucleic acids comprising SEQ ID NO:4; and

(b) nucleic acid comprising a 285 bp amplification product made by PCR amplification using primers SEQ ID NO:1 and SEQ ID NO:2.

33. A method according to claim 22 wherein the genus is *Nonomuria*.

34. A method according to claim 33 further comprising the step of lysing bacteria in the sample prior to step (a).

35. A method according to claim 34 wherein the probe is a radioactively labeled probe.

36. A method according to claim 35 wherein hybridization conditions are stringent hybridization conditions.

37. A method according to claim 36 wherein said probe comprises nucleic acids selected from the group consisting of:

(a) nucleic acids comprising SEQ ID NO:5; and

(b) nucleic acid comprising a 285 bp amplification product made by PCR amplification using primers SEQ ID NO:1 and SEQ ID NO:2.

38. A method according to claim 22 wherein the genus is *Planobispora*.

39. A method according to claim 38 further comprising the step of lysing bacteria in the sample prior to step (a).

40. A method according to claim 39 wherein the probe is a radioactively labeled probe.

41. A method according to claim 40 wherein hybridization conditions are stringent hybridization conditions.

42. A method according to claim 41 wherein said probe comprises nucleic acids selected from the group consisting of:

(a) nucleic acids comprising SEQ ID NO:6; and

(b) nucleic acid comprising a 285 bp amplification product made by PCR amplification using primers SEQ ID NO:1 and SEQ ID NO:2.